REMARKS

I. Status of the Claims

Claims 41-63 are pending in the instant application. Claim 41 has been amended, without prejudice or disclaimer, by adding the following proviso "provided that when Y is $-(CH_2)_m-$ and m is 0, that at least one of R^5- R^9 is other than a hydrogen." The proviso was added to exclude the compound taught by Gronowitz et al. (Arkiv Kemi, 28, 587 (1967)). According to the C.C.P.A., adding a proviso to a claim to overcome the prior art does not introduce new matter into the claim. See *In re Johnson* (*Johnson*), 194 U.S.P.Q. at 187. In *Johnson*, the court stated that claiming less than the full scope of a disclosure is a perfectly legitimate procedure since it is for an inventor to decide what bounds of protection he will seek. *Id.* at 195-196. In the present case, the amendment to Claim 41 merely claims less than the full scope of the disclosure and therefore does not add new matter.

II. Withdrawal of Rejection of Claims 41 and 43-51 Under 35 U.S.C. §112

Applicants acknowledge and thank the Examiner for withdrawing the following rejections: the rejection of Claim 41 under 35 U.S.C. §112, first paragraph as new matter and the rejection of Claims 43-51 under 35 U.S.C. §112, second paragraph as indefinite.

III. Rejection of Claims 41-51 and 53-63 Under 35 U.S.C. §112, First Paragraph

The Examiner has maintained her rejection of claims 41-51, and 53-63 under 35 U.S.C. §112, first paragraph, as allegedly not reasonably providing enablement for the preparation and use of compounds other than those having substituents R^5 (or R^6) as a quinolinyl ring while R^7 and R^8 are hydrogen-i.e., compound 26-6. See Office Action pages 2-3.

The Examiner alleges that the specification provides the guidance for preparing only a limited set of pyrimidinyl compounds which are substituted with a quinolinyl ring because the specification requires intermediate 5-7, which is specific to compound 26-6. Id. at 3. The Examiner acknowledges that the specification provides other functional groups, and/or rings as intermediates represented by one of R^5 -R^8 but the Examiner takes the position that these intermediates are used in the preparation of naphthyridinyl compounds rather than pyrimidinyl compounds and there is no suggestion that these intermediates can be used in place of intermediate 5-7 to make other pyrimidinyl compounds.

Applicants respectfully traverse this rejection for the reasons previously discussed in the Office Action Response filed May 14, 2003, in addition to those presented below.

Applicants assert that the specification fully satisfies the enablement requirement of 35 U.S.C. 112, first paragraph, thus enabling the claims and providing ample guidance to prepare compounds having various substituents defined by R^5–R^8.

Applicants respectfully disagree with the Examiner's contention that the specification fails to suggest that intermediate 5-7 taught in Example 26 can be replaced with other intermediates disclosed in the various examples to make other pyrimidinyl compounds--not just compounds comprising a quinolinyl moiety. The specification clearly states that compound 26-6 was synthesized by the coupling of 26-5 and 5-7 in the same manner as described for 1-6 and 1-7 to furnish 1-9. See Specification, pg 152, lines 30-31. Compound 1-9 comprises a naphthyridinyl terminus which corresponds to 26-6's pyrimidinyl terminus and a pyridinyl moiety which corresponds to 26-6's quinolinyl moiety. The skilled artisan, having knowledge of general synthetic chemistry, would reasonably expect that compound 26-6 can be modified by replacing the quinolinyl group with a pyridinyl moiety.

Regarding the Examiner's observation that different combinations of reagents were used for the addition of intermediate 5-7 to a naphthyridinyl containing versus a pyrimidinyl containing compound, Applicants point out that these reagent variations are in the purview of a skilled artisan and would not require undue experimentation to implement. In organic synthesis, it is possible to use more than one type of reagent (protecting group, solvent, etc.) during a synthesis process while still achieving the same desired coupling effect. For example, compound 6-6 was used as a coupling intermediate for compounds 6-7, 14-5, and 14-6. See Specification, pages 83 and 112, respectively. During the coupling process, two different protecting groups, HOAT and HOBT, were utilized to make 6-7 and 14-5, respectively. Id. A skilled artisan would realize that certain protecting groups such as HOAT and HOBT are commonly used and serve similar functions. See for example, Greene, T. et al, Protective Groups in Organic Synthesis, John Wiley & Sons, 2002. It is reasonable to expect the skilled artisan could substitute HOBT with HOAT.

Further, other exemplified compounds having moiety substitutions in the same position as the quinolinyl group of compound 26-6 indicate that these moieties can be attached to the core structure in a similar manner to that described in Examples 1 and 26. The coupling reaction of 26-5 and 5-7 outlined in Scheme 26 is a coupling of a carboxylic acid with an amine. Similar coupling reactions of carboxylic acids to various amines are disclosed in the present specification. Many examples of this coupling require a protecting group (BOP, HOBT, HOAT), and least one coupling catalyst (1-(3-dimethylaminopropyl)-3-ethylcarboamide·HCL (EDC) or N-methylmorpholine (DMM)). See for example, Schemes 1, 3, 4, 5, 6, 7, 9, 10 (compound 9-1),

12, 14, 17, 21, 26, 27, and Scheme A (A-2). It should be noted that with the exception of 26-6, the final compounds exemplified in these schemes have terminal moieties that are not pyrimidinyl. Other Schemes in the specification illustrate different well known coupling techniques. See for example, Schemes 8, 11, and 16. As such, one of ordinary skill in the art would reasonably expect that the moieties exemplified in these schemes could be attached to a core platform having a pyrimidinyl terminus utilizing analogous synthesis techniques.

Applicants would like to point out that EDC is a recognized coupling catalyst and is listed in the Aldrich Catalog as such. See Attachment A. NMM is also a known catalyst. In addition, techniques for the coupling of a carboxylic acid to an amine are well known in the art. See for example, O. Marder's "industrial application of coupling reagents in peptides" (Attachment B), and Sigma Aldrich's "Peptide Synthesis" (Attachment C). Applicants submit that a skilled artisan, armed with commonly known organic synthesis techniques and guided by the present specification, could make and use the compounds of the present invention without undue experimentation.

The Examiner alleges that exemplification of only one pyrimidinyl compound encompassed in a broad genus is insufficient to satisfy the enablement requirement of 35 U.S.C. 112, first paragraph due to the unpredictability of the chemical arts thus leading to the prospect of undue experimentation. Office Action, page 4. Applicants respectfully disagree because the enablement requirement of 35 U.S.C. 112, first paragraph does not require that every species encompassed by the claims, even in unpredictable arts, be disclosed. See *In re Angstadt*, 537 F.2d at 503, 190 U.S.P.Q. at 218. Additionally, in determining undue experimentation, a considerable amount of experimentation is permissible, if it is merely routine, or if the specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. See MPEP §2164.06 and *In re Wands*, 858 F.2d 731, 737.

The specification includes 115 pages of extensive experimental direction providing over 85 working examples describing synthesis techniques analogous to those described in Examples 1 and 26. In addition, Example 26 discloses that the techniques utilized for compounds having a napththyridinyl terminus can be implemented for compounds having a pyrimidinyl terminus. The specification also incorporates by reference at least eight documents disclosing various synthesis techniques that can be employed to make the compounds of the present invention. See Specification, p. 66. Additionally, coupling techniques disclosed in the present application are commonly known in the art. Based upon the guidance found in the specification coupled with commonly known synthetic organic chemistry techniques, undue experimentation is not necessary to arrive at the compounds of the present invention, because a

skilled artisan would reasonable expect that the synthesis techniques disclosed in the specification could be utilized to make the claimed compounds by analogous techniques.

The Examiner has not made a prima facie case of non-enablement, and in light of the arguments presented, it is not reasonable to conclude that Applicants have not enabled the claims. Accordingly, Applicants respectfully request the rejection of claims 41-51, and 53-63 under 35 U.S.C. §112, first paragraph be withdrawn.

IV. Rejection of Claims 41 and 42 Under 35 U.S.C. §102(b)

The Examiner has rejected Claims 41 and 42 under 35 U.S.C. §102(b), as allegedly anticipated by Gronowitz S., et.al., Arkiv Kemi, 28, 587-601 (1967).

Gronowitz et. al discloses compound 5-(4-pyrimidyl)-n-valeric acid. Applicants submit that the rejection under U.S.C. §102(b) has been obviated in light of the amendment to claim 41. As amended, Claim 41 does not encompass 5-(4-pyrimidyl)-n-valeric acid. As such, Gronowitz et. al. fails to anticipate the presently claimed invention.

Additionally, Gronowitz et.al. fails to render the presently claimed invention obvious. Gronowitz et al. is directed to substitution reactions of 2- and 3-(4-pyrimidyl) thiophenes and is completely silent as to any pharmacological properties of the compound. There is nothing in Gronowitz et. al to motivate one of ordinary skill in the art make or use the compounds of the present invention.

As Gronowitz et. al, fails to teach or suggest the compounds of the present invention, Applicants respectfully request the rejection of claims 41 and 42 under 35 U.S.C. §102(b) be withdrawn.

V. Rejection of Claims 41-51 and 53-63 under Obviousness-Type Double Patenting

In the Office Action mailed March 28, 2003, the Examiner provisionally rejected Claims 41-51 and 53-63 under the judicially created doctrine of obviousness-type double patenting as allegedly unpatenable over claims 1-6, and 9-16 of Application No. 09/767,471. Subsequent to the issuance of the rejection in this application, Claim 8 of U.S. Application No. 09/757,471 was allowed and Claims 1-6, and 9-16 were cancelled. The claimed subject matter of allowed U.S. Patent Application No. 09/757,471 does not overlap with the presently claimed subject matter. A divisional application, No. 10/618,414 was filed on July 10, 2003.

As the doctrine of obviousness-type double patenting is concerned with overlapping claimed subject matter and there is no conflicting claimed subject matter between the presently claimed invention and the allowed claim of U.S. Application No. 09/757,471. Thus

the obviousness-type patenting rejection is improper. Accordingly, Applicants respectfully request that this obviousness-type double patenting rejection be withdrawn.

VI. Allowable Subject Matter

Applicants thank the Examiner for her continued indication that Claim 52 would be allowable if rewritten in independent form.

CONCLUSION

In view of the foregoing remarks, Applicants respectfully request the reconsideration of the pending claims and the reexamination of the application. The timely allowance of the pending claims is respectfully requested.

If a telephonic communication with the Applicants' representative will advance the prosecution of the instant application, please telephone the representative indicated below. Applicants believe no additional fees are due but the Commissioner is authorized to charge any fees required in connection with this response to Merck Deposit Account No. 13-2755.

Respectfully submitted,

By Patricia A. Shatynski

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Rahway, New Jersey 07065-0907
(732) 594-1652

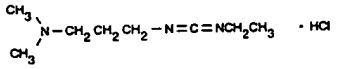
Date: December 15, 2003

Attachments A-C

ATTACHMENT A

■ Dimethylam ■

			$
30,682-7	***trans,trans*-5-[4-(Dimethylamino)phenyl]-2,4-pentadienal**, 99% [*20432-36-4*] $(CH_3)_2NC_6H_4CH=CHCH=CHCHO$ FW 201.27 mp 155-156° *FT-NMR* 1(2),931C *SI* 271,C,6 *R&S* 1(2),1713A	10mg 25mg	48.65 84.65
21,021-8 NEW ★	**3-(Dimethylamino)-1,2-propanediol**, 98% [*623-57-4*] $(CH_3)_2NCH_2CH(OH)CH_2OH$ FW 119.16 bp 216-217° n_D^{25} 1.4609 d 1.004 Fp 221°F(105°C) $[\alpha]_D^{25}$ 0° *Beil.* 4,302 *FT-NMR* 1(1),549C *FT-IR* 1(1),345D *SI* 52,C,3 *Safety* 2,1342A *R&S* 1(1),369H *CORROSIVE*	5mL 25mL	10.75 36.00
D14,420-7 ★	**1-Dimethylamino-2-propanol**, 99% [*108-16-7*] $(CH_3)_2NCH_2CH(OH)CH_3$ FW 103.17 bp 121-127° n_D^{25} 1.4193 d 0.837 Fp 95°F(35°C) *FT-NMR* 1(1),546C *FT-IR* 1(1),343A *SI* 51,C,9 *Safety* 2,1342B *R&S* 1(1),367J *RTECS#* UB3150000 *FLAMMABLE LIQUID CORROSIVE*	50mL 250mL 1L	12.00 15.30 36.70
D14,440-1 ★	**3-Dimethylamino-1-propanol**, 99% [*3179-63-3*] $(CH_3)_2N(CH_2)_3OH$ FW 103.17 bp 163-164° n_D^{25} 1.4360 d 0.872 Fp 97°F(36°C) *Beil.* 4(1),433 *FT-NMR* 1(1),546A *FT-IR* 1(1),342B *SI* 51,A,9 *Safety* 2,1342C *R&S* 1(1),367H *FLAMMABLE LIQUID IRRITANT*	5mL 100mL 500mL	11.65 15.00 45.55
	1-(Dimethylamino)-2-propanone, see 10,769-7, (Dimethylamino)acetone page 567		
23,448-6 ★	**3-(Dimethylamino)propionitrile**, 98% [*1738-25-6*] $(CH_3)_2NCH_2CH_2CN$ FW 98.15 mp -43° bp 171°/750mm n_D^{25} 1.4260 d 0.870 Fp 145°F(62°C) *Beil.* 4(3),1265 *FT-NMR* 1(1),1382A *FT-IR* 1(1),859C *SI* 157,D,3 *Safety* 2,1342D *R&S* 1(1),1007F *RTECS#* UG1575000 *HIGHLY TOXIC CORROSIVE*	5mL 100mL 500mL	12.65 14.90 42.65
D14,480-0 ★	**3-Dimethylaminopropiophenone hydrochloride**, 99% [*879-72-1*] $C_6H_5COCH_2CH_2N(CH_3)_2 \cdot HCl$ FW 213.71 mp 153-154° *Beil.* 14(2),38 *FT-IR* 1(2),47A *SI* 260,C,8 *Safety* 2,1343A *R&S* 1(2),1653H *RTECS#* UH0600000 *TOXIC IRRITANT*	25g 100g	16.45 43.60
29,355-5	**4-(3-Dimethylaminopropoxy)benzaldehyde**, 96% [*26934-35-0*] $(CH_3)_2N(CH_2)_3OC_6H_4CHO$ FW 207.28 n_D^{25} 1.5430 d 1.031 Fp >230°F(110°C) *FT-NMR* 1(2),944C *FT-IR* 1(3),1292D *SI* 273,D,1 *Safety* 2,1343B *R&S* 1(2),1719E *IRRITANT*	1g 5g	16.65 55.55
41,565-0 NEW ★	**3-(Dimethylamino)propyl acrylate**, 95% [*18526-07-3*] $H_2C=CHCO_2(CH_2)_3N(CH_3)_2$ FW 157.22 bp 167° n_D^{25} 1.4400 d 0.928 Fp 155°F(68°C) *SI* 120,D,3 *CORROSIVE*	5mL 25mL	22.25 74.25
24,005-2 ★	**3-Dimethylaminopropylamine**, 99 + % [*109-55-7*] (*N,N*-dimethyl-1,3-propanediamine) $(CH_3)_2N(CH_2)_3NH_2$ FW 102.18 bp 133° n_D^{25} 1.4350 d 0.812 Fp 60°F(15°C) *Beil.* 4(3),554 *Fieser* 1,274 *FT-NMR* 1(1),494B *FT-IR* 1(1),306A *SI* 45,A,3 *Safety* 2,1343C *R&S* 1(1),327J *RTECS#* TX7525000 *FLAMMABLE LIQUID CORROSIVE*	50mL	15.05
D14,500-9 ★	**3-Dimethylaminopropylamine**, 99% [*109-55-7*] (*N,N*-dimethyl-1,3-propanediamine) $(CH_3)_2N(CH_2)_3NH_2$	25mL 500mL 1L	13.15 14.45 20.80
D14,520-3 ★	**3-Dimethylaminopropyl chloride hydrochloride**, 96% [*5407-04-5*] (3-chloro-*N,N*-dimethylpropylamine hydrochloride) $(CH_3)_2N(CH_2)_3Cl \cdot HCl$ FW 158.07 mp 141-144° *Beil.* 4,148 *FT-IR* 1(1),326B *SI* 48,C,9 *Safety* 2,1343D *R&S* 1(1),353B *RTECS#* UI0209950 *IRRITANT HYGROSCOPIC*	25g 100g 500g	5.30 13.60 51.15
	5-(3-Dimethylaminopropyl)-10,11-dihydro-5*H*-dibenz[*b,f*]azepine hydrochloride, see 28,626-5, Imipramine hydrochloride page 845		
16,146-2 ★	**1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride**, 98 + % [*25952-53-8*] FW 191.71 mp 111-113° *Fieser* 12,199 *FT-IR* 1(1),877A *SI* 160,C,8 *Safety* 2,1344A *R&S* 1(1),1035F *RTECS#* FF2200000 *MOISTURE-SENSITIVE IRRITANT* Coupling reagent used to form amide bonds[1] in oligonucleotides[2] and tetraazamacrocycles.[3] (1) *Tetrahedron Lett.* **1993**, *34*, 7685. (2) *ibid.* **1993**, *34*, 6189. (3) *Bioconjugate Chem.* **1992**, *3*, 108.	1g 10g	11.85 55.30
16,534-4	**1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide methiodide** [*22572-40-3*] $C_2H_5N=C=N(CH_2)_3N(CH_3)_3I$ FW 297.18 mp 97-99° *FT-NMR* 1(1),1413C *SI* 160,D,8 *Safety* 2,1344B *R&S* 1(1),1035E *MOISTURE-SENSITIVE IRRITANT* Water-soluble carboxyl modifying reagent for proteins. *Biochemistry* **1992**, *31*, 9090.	1g 10g	12.00 67.25
40,947-2 ★	***N*-[3-(Dimethylamino)propyl]methacrylamide**, 99% [*5205-93-6*] $H_2C=C(CH_3)CONH(CH_2)_3N(CH_3)_2$ FW 170.26 bp 134°/2mm n_D^{25} 1.4790 d 0.940 Fp >230°F(110°C) *SI* 136,C,2 *IRRITANT LIGHT-SENSITIVE*	250mL 1L	14.90 41.25
	***N*-[3-(Dimethylamino)propyl]methacrylamide, methyl chloride quaternary salt**, see 28,065-8, [3-(Methacryloylamino)propyl]trimethylammonium chloride page 936		



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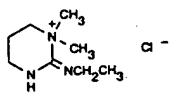

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ATTACHMENT B

Industrial application of coupling reagents in peptides

OLEG MARDER [1]
FERNANDO ALBERICIO [2,3]

ABSTRACT

Peptides play a key role in the post-genomic and proteomic era. From an industrial basis, the formation of the amide bond is crucial for obtaining an efficient and economic process. Mechanisms associated with the different methods are reviewed. Cl-HOBt as additive and HCTU/TCTU are excellent alternatives to the most classic coupling methods.

INTRODUCTION

There is no doubt that the last years are recognised as the renaissance of the peptide world. The important advances in molecular biology and gene technology are bringing significant changes in biomedical sciences. The elucidation of the human genome, followed by advancements in functional genomics and proteomics, will revolutionize our understanding of the detailed molecular mechanisms, underlying a broad spectrum of diseases. These developments will also identify new therapeutic targets and suggest novel mechanism-based therapeutic paradigms. Peptides will play a key role in all these processes.

There has been a notable increase in research institutions and pharmaceutical companies involved in the development of peptide-based drugs, resulting in an increase in the number of university core facilities and spin-offs, commercial peptide suppliers, contract peptide manufacturers, and other outsourcing institutions.

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Tel Aviv, Israel

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E-08028 Barcelona, Spain

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Martí i Franqués 1-11
E-08028 Barcelona, Spain

In the actual pharmaceutical market, peptides are not only considered as hormones, as in the past, but also as active pharmaceutical ingredients (API), in antibiotics, antiviral and in other therapeutic areas such as cancer as immunomodulators and anti-angiogenesis agents, CNS and neurogogical disorders as analgetics and anti-obesity drugs, immune disorders for the treatment of allergy, asthma and autoimmune disease (1).

Furthermore, over 200 new peptide-based drugs are under different stages of development with 50% of them under clinical trials and prior to approval (2). Nowadays, peptides represent 1% of total API with a market of US$ 300-500 M per year and a growth rate of 15-25% annually (1), with the expectation of a 100% increase in the next two years when generic and recently approved new chemical entities enter the market (3). This is reflected by the fact that over ten known bulk peptide producers and over twenty companies are offering custom peptides synthesis and these numbers, as well as capacity are growing (4). This trend has subsequently affected all raw material manufacturers and the entire peptide industry supply chain.

Today, manufacturing companies face the unprecedented challenge of production of hundred kilograms to tons quantities of complex peptides involving modern technologies. Thus, the customer supplier relationships are becoming more complex, involving basic producers with their extensive knowledge and experience in the reagents field and increasingly into a process development at different stages of production.

A key step in the peptide production process is the formation of the peptide bond. This requires the activation of a carboxylic acid, which is usually carried out using the so-called **peptide coupling reagents** (5,6). In addition to peptides, amide bonds are present in a huge array of other organic compounds of biological interest such as peptoids, oligocarbamates, oligoamides, β-lactams, polyenamides, benzodiazepines, diketopiperazines, and hydantoins. Furthermore, the ester group is another important functionality present in many organic compounds and can also be prepared directly from the carboxylic acid using **peptide coupling reagents**.

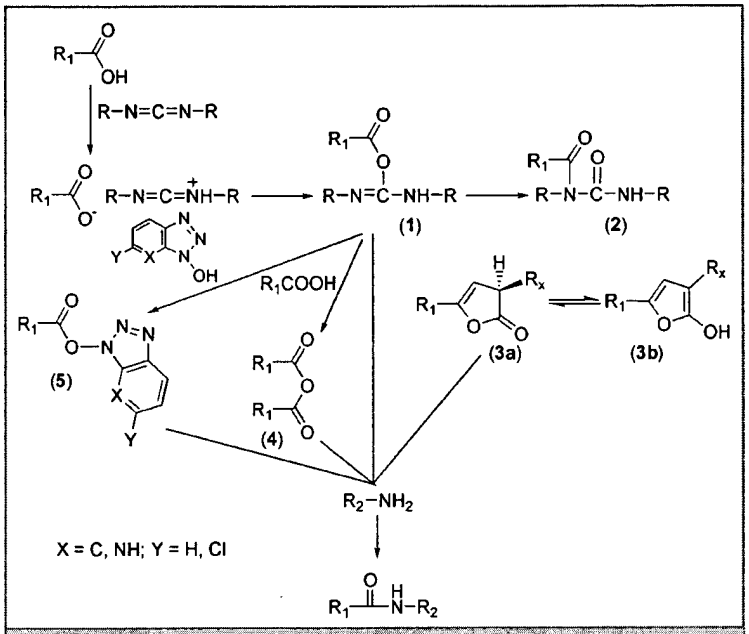

Figure 1 - Mechanism of peptide bond formation through carbodiimide activation

Although the synthesis of medium-large peptide for basic research is a well established procedure, the combination of the 20 proteinogenic amino acids and growing number of unnatural amino acids makes each peptide synthesis at the industrial level unique, requiring closer attention to each amino acid coupling. Some of the rules for coupling reagents validated in the research scale can be applied at industrial level, but the results are still hardly predictable. Therefore, although peptides are produced in the industry in hundred kilograms to tons scale, there is still a need to search for the ultimate **coupling reagent**.

The **peptide coupling reagent** field has clearly evolved in the last decade from carbodiimides to onium (phosphonium and uronium) salts. The era of industrial coupling reagents began in 1955 with the introduction of dicyclohexylcarbodiimide (DCC) (7), which at that time was already known and well studied, as a reagent for formation of amide bond (8). Unfortunately, carbodiimides did not comply with the concept of ultimate coupling reagents because its high reactivity provokes racemization and side reactions during the coupling reaction (Figure 1).

The mechanism of the carbodiimide activation, which is complex and depending on the solvent, starts by a proton transfer, followed by addition of the carboxylate to form the *O*-acylisourea (**1**). This is the most reactive specie that can attack the amino component to give the corresponding amide. However, the *O*-acylisourea (**1**) can undergo a rearrangement to give the *N*-acylurea (**2**), which is not reactive, or sustain an intramolecular cyclization to give a 5(4*H*)-oxazolone (**3**), which is less reactive than **1** and can tautomarize with the corresponding loss of chirality. If activation is carried out in a solvent of low dielectric constant such as $CHCl_3$ or CH_2Cl_2, the formation of **1** occurs instantaneously, which is absent of a nucleophile or base and can be stable for many hours. However, if the activation is carried out in a more polar solvent such as DMF, no immediate reaction can be detected, and a complex mixture of starting amino acid, symmetrical anhydride (**4**), and **2** is formed. If the activation is carried out in the presence of an extra equivalent of acid, **4** is formed, which is also very reactive.

At the beginning of the 70's, 1-hydroxybenzotriazole (HOBt) (9) was proposed as an additive to DCC to reduce racemization and from then on other benzotriazole derivatives such as 1-hydroxy-5-chlorobenzotriazole (Cl-HOBt) (10) or 1-hydroxy-7-azabenzotriazole (HOAt) (11) have also been used. The OBt active esters (**5**) are less reactive than **1**, but are more stable and less prone to racemize. All these factors make the addition of benzotriazole derivatives almost mandatory to preserve the peptide bond formation by carbodiimide activation of low yields and undesired side reactions.

In the last decade onium (phosphonium and aminium/uronium) salts of hydroxybenzotriazole derivatives have been introduced. Although, they have been rapidly adapted for research purposes, only a few of them have been found compatible with current industrial requirements and synthetic strategies and therefore adopted by the industry.

The specie that reacts with onium salts is the carboxylate (Figure 2) and therefore the presence

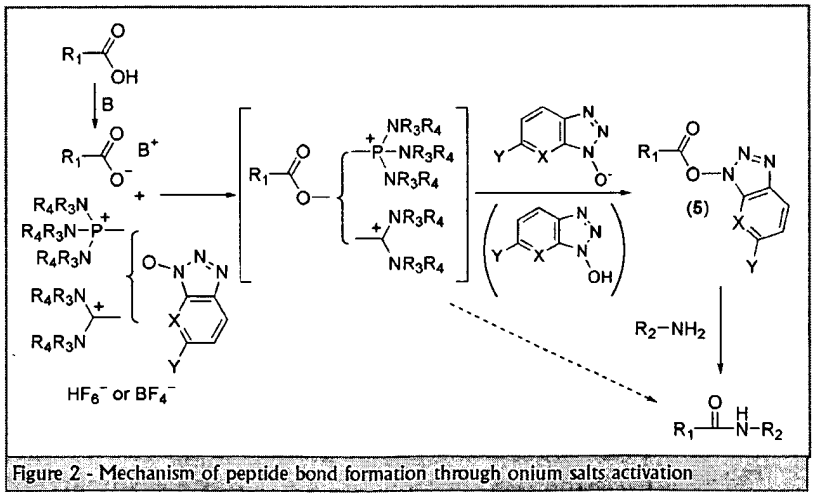

Figure 2 - Mechanism of peptide bond formation through onium salts activation

of at least one equivalent base is essential. The intermediate species acyloxy-phosphonium or amidinium salts have not been detected and react immediately with the benzotriazole derivative (an extra equivalent of it is added in some synthetic protocols) to give **5**, which react with the amino component to give the corresponding amide.

Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977–983.

The following additional abbreviations are used:

Cl-HOBt, 6-chloro-1-hydroxybenzotriazole;

DCC, *N,N'*-dicyclohexylcarbodiimide;

DIPCDI, *N,N'*-diisopropylcarbodiimide;

DMF, *N,N*-dimethylformamide;

EDC, 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide;

Fmoc, 9-fluorenylmethoxycarbonyl;

HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo [4,5-*b*]pyridino-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate;

HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide;

HCTU, *N*-[(1*H*-6-chlorobenzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide;

HOAt, 7-aza-1-hydroxybenzotriazole;

HOBt, 1-hydroxybenzotriazole;

PyAOP, 7-azabenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino) phosphonium hexafluorophosphate;

TNBSA, trinitrobenzenesulfonic acid;

TBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino) methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide;

TCTU, *N*-[(1*H*-6-chlorobenzotriazol-1-yl) (dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide;

TFA, trifluoroacetic acid;

Amino acid symbols denote the L-configuration unless stated otherwise.

WHAT IS A GOOD COUPLER FROM THE INDUSTRIAL POINT OF VIEW?

Effective Coupling Reagent

- Works with high efficiency for a wide variety of peptide sequences.
- Works in steochimetric quantities.
- Has a high conversion rate at room temperature.
- Works for both solution and solid-phase peptide synthesis.
- Is soluble in all the currently used solvents and can be used at high concentrations.
- Solutions are stable for several days at room temperature.
- Allows monitoring of coupling reagents.
- Shows few side reactions.
- Its secondary products after coupling can be completely removed by solvent extraction.

Cost-Effective Reagent

Can Be Produced in Large Quantities

- Conventional raw material available.
- Technology is known and does not require installation of sophisticated equipment.
- Chemistry is adaptable for up-scaling.
- Raw material solvents and catalysts are safe for the producer.
- Effective waste management.
- Has a prolonged shelf-life at ambient conditions.

Safe for Producer, User, and Environment

- Material is not toxic, corrosive or self-reactive.
- The use of material does not result in formation of toxic by-products.
- Does not generate hazardous waste.
- Can be disposed with low pollution risk.

THERE ARE MORE THAN 80 REAGENTS KNOWN TODAY, HOWEVER ONLY A FEW HAVE FOUND THEIR WAY TO THE INDUSTRY

Analyzing the bulk coupling reagent market, we can see that is shared between three main groups of coupling reagents in addition to the additives (Figure 3).

Additives

As was outlined in the introduction, the addition of benzotriazoles [HOBt (**6**) and Cl-HOBt (**7**)] to the carbodiimides based coupling reagents leads to the formation of the benzotriazole active esters that are less reactive than the *O*-acylisourea (**5**), reducing racemization of the protected amino acid and avoiding the formation of other derivatives less reactive. Cl-HOBt (**7**) performs at least as well as HOBt (**6**), but since it is more acidic (pK_a: 3.35 for Cl-HOBt and 4.60 for HOBt) it is a better leaving group and its active esters are more reactive than OBt esters. As discussed in section 5, another advantage of Cl-HOBt is that the chlorine atom stabilized the structure, making

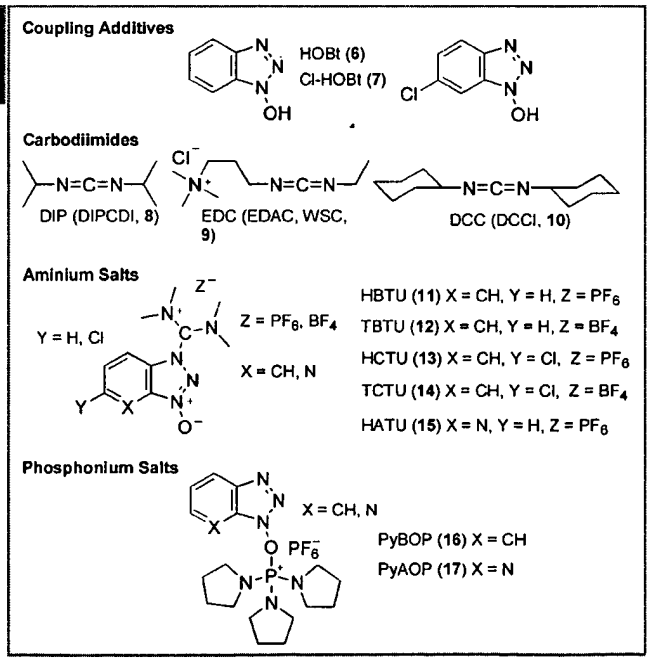

Figure 3 - The most common coupling reagents and additives used in the industry

Cl-HOBt a less hazardous reagent. Although its concourse is not strictly necessary as shown in Figure 2, HOBt and Cl-HOBt are also added to the aminium salts mediated coupling reactions, with the purpose of favouring the active ester formation. Thus, Cl-HOBt is used as an additive together with HBTU to suppress racemization during fragment condensation assay in the industrial synthesis of Fuzeon (T-20) (12).

The pyridine derivative of HOBt (HOAt) is not for industrial use, because the extra nitrogen in the phenyl ring makes the structure unstable (see section 5b).

Coupling Reagents

1. Carbodiimide Coupling Reagents [DIPCDI (8), EDC (9), DCC (10)]

Carbodiimides are the cheapest coupling reagents and their primary active species [*O*-acylisourea (**5**)] one of the most reactive. Problems associated with their use are racemization and low yields due to the formation of the poorly active *N*-acylurea (**2**). Use of solvents of low dielectric constant such as $CHCl_3$ or CH_2Cl_2 minimize both reactions. Although, the use of these solvents is precluded in the automatic solid-phase mode at the research level due to specific characteristics of the synthesizers, they can be used in manual or semi-automatic modes. Addition of benzotriazole derivatives also minimizes side-reactions. DCC (**10**) is incompatible with Fmoc/*t*Bu solid-phase chemistry, because the dicyclohexylurea is not soluble is common solvents. In solution chemistry, ureas are always difficult to be removed and water-soluble carboddiimide [EDC (**9**)] is a useful alternative.

When carbodiimides are used with two equivalents of protected amino acids, the symmetrical anhydride (**4**) is formed, which is a very reactive, active specie. This method has some economical consequences, because a double amount of protected amino acids are required.

2. Aminium Salts of Benzotriazoles

The use of the most reactive aminium salt, HATU (**15**) (11), is inconvenient because of the price, which makes its use detrimental for industry. HCTU (**13**)/TCTU(**14**) (13) are a good alternative to HBTU (**11**)/TBTU (**12**) (14), because the presence of the Cl-HOBt makes those reagents more reactive.

During the activation of hindered carboxylic components, such as those involved in cyclization reactions of coupling of hindered amino acids, the aminium salts can react with the amino component, leading to a guanidine derivative, a process that terminates the peptide chain. Recently, it has been discovered that aminium salts can contain traces of dimethylamine, which can also react with the carboxylic component to give the corresponding dimethylamide.

Aminum salts having the counterion tetrafluoroborate are more soluble than hexafluoroborate salts, which allow preparation of more concentrated solutions.

3. Phosphonium Salts of Benzotriazoles

As occurs with the aminium salts, the use of PyAOP (**16**) (15), which is derived from HOAt, is the most reactive phosphonium salt, but it is prohibitive for industrial purposes due to its extremely high price. The benzotriazole derivative, PyBOP (**15**) (16), which is also more expensive than its aminium analogue, is specially useful for cyclization steps or for the activation of hindered amino acids, where the use of aminium salts can lead to the formation of guanidine derivatives. For the same reason, PyBOP can be used in excess and added during the coupling step, because it will not terminate the peptide chain.

These derivatives can contain pyrrolidine, which can also react with the carboxylic acid giving the corresponding pyrrolidide derivative.

WHY CLASSIC ACTIVATORS WILL REMAIN FOR DECADES IN THE MARKET?

Despite the presence of novel, highly effective coupling reagents, the classical activators have remained in the market for years. We could point out a few reasons to define this fact:

- Influence of the human factor, which includes inertness or unavailability of human recourses to test and evaluate new coupling systems.
- Marketing problem, when a manufacturing company or distributor could not identify and localize its potential target group and end users.
- Supply chain when the information about new products and application provided by developer or supplier does not always can find its addressee.
- Legal and secrecy issues do not always give the opportunity of knowledge exchange between peptide contract manufacturer and raw material producer.
- Role of existing regulation requirements. List of the reagents used in a synthesis and synthetic process and methods used in a synthesis, including the reagents and synthesis condition should come as a part of an Investigational New Drug application (IND) for Phase I study (17), of a NDA (New Drug Application) (18) submitted to FDA at the completion of phase II clinical trial, and of a DMF (Drug Master File). The changes in manufacturing process and reagents used should be introduced before submission of NDA. The post approval changes in manufacturing process can be submitted and approved by FDA (Food and Drug Administration), together with additional tests confirming that such changes had no effect on identity, quality, bioavailability, toxicity and even efficiency of new drug substance. That would cause a delay in product approval and will tremendously affect cost of development of new drug substance. Process development chemist should present final manufacturing process that should be up-scalable and cost effective at an early stage of drug development. The close connection of development chemist with manufacturer of raw and source materials at an

early pre-clinical stage would help to avoid any post-approval changes in manufacturing process.

USING HIGH QUALITY, SAFE AND COST EFFECTIVE COUPLERS AT THE EARLY STAGE OF PROCESS DEVELOPMENT

The use of efficient coupling reagents is an effective way of cost saving at production stage and de-bottlenecking at purification step.

a. Contaminant and Impurities During Peptide Synthesis

Epimerised (Racemised) Peptides

The presence of these side products is strongly tied to the coupling method. Thus, efficient coupling reagents in combination with racemization suppressor additives and solvents of low dielectric constant should minimize the formation of epimers. Furthermore, reducing the time of preactivation of the carboxylic acid could also reduce the racemization. The presence of these peptides is very often difficult to detect, because their chromatographic behavior is similar to the target peptide.

Deletion Peptides

As above, the use of the most efficient coupling reagents should minimize the deletion of peptide formation. In solid-phase, the use of more than one analytical method to detect the presence of free amines is advisable. Although, ninhydrine test is the broader method used (19), very often it gives a false negative. Trinitrobenzenesulfonic Acid (TNBSA) (20) and especially NF-31 (21) , are excellent alternatives to the ninhydrine test.

Truncated Peptides

In solid-phase mode, truncated peptides are often formed by precipitation of the peptide. This phenomenon occurs when the resin is overdried. For this reason, it is advisable to leave the resin wet with solvent between the different synthetic steps.

Terminated Peptides

The presence of acetic and even of trifluoroacetic acid traces can terminate the peptide chain. Furthermore, as discussed above, aminium salts can also guanilidate free amine functions.

Modified Peptides

Aspartiimides that can lead to both α and β peptides are difficult to detect. The formation of aspartiimides, which can take place during basic treatments (*e.g.* during piperidine treatment in a Fmoc/*t*Bu strategy) can be reduced by adding HOBt.

Other important modifications can take place during the final deprotection/cleavage step. This can be minimized by the using of the appropriate scavengers.

Reaction By-Products

Ureas from carbodiimides and aminium salts, phosphotriamides from phosphonium salts, coupling additives, and scavengers used in the last step are the most important reaction by-products.

Oxidized Peptides

These kinds of peptides are mainly encountered in sulfur-based peptides and are unrelated to the coupling reagent used.

b. Toxic and Hazardous Reagents Used in Synthesis

Up-scaling from the bench and beyond requires from the manufacturer reevaluation of very critical parameters such as transportation, storage, user safety and waste management of raw materials and in particular coupling reagents. There are situations where reagents, convenient and handy in laboratory, become unmanageable by contract manufacturer when it comes to bulk peptide manufacturing. This may lead to production downscaling and reevaluation of synthetic procedure. The transportation and subsequently storage and use issue, becomes critical for the reagents containing the imidazole ring benzotriazoles (HOBt), as well as for reagents with an extra nitrogen in the phenyl ring (HOAt), which according to recent studies make the structure unstable with relatively high sensitivity to friction, spark, and electrostatic discharge resulting in burning or explosion. However the onium salt component (HBTU, TBTU, HATU, PyBOP), and derivatization of the phenyl ring with halogen ion (HCTU, TCTU) stabilize the structure (13). It is shown that addition of water or solvents can also make the compound less sensitive. Reagents can still be transported and stored to comply with international transportation regulations for hazardous substances, but the bulk use of some of them is in question, since the additional safety and logistic measures should be considered. This may include the safety revaluation of existing equipment following installation of an additional gear in production site, antistatic flooring in storage facilities and production site and personnel training. Logistic and transportation department control material packed in appropriate containers should not exceed weight restrictions and appropriate labeling and documentation.

Another important issue is the toxic properties of substances used in bulk peptide synthesis. The modern chemical laboratories are usually well equipped to provide environmental and personal protection to technicians and researchers from possible toxic effect of raw material. In general, chemical fume hood, laboratory coat, gloves and facemask usually gives necessary protection, yet a number of researchers have reported the adverse effect of certain coupling reagents and additives on their physical condition.

Carboxydiimides for example is well known for their skin irritating properties while prolonged use some of benzotriazole based coupling reagents and additives (HOBt, HBTU, TBTU) may not only cause skin irritation and contact dermatitis, but also sensitization and allergic reaction of respiratory tract (22). The situation becomes more complicated when it comes to industry. To protect the worker and the environment, special precautions should be taken by manufacturer. This includes installation of HEPA filters, special equipment for preventing dusty conditions at the time of reactor loading, personal protection which usually consists of protective suit, gloves, boots and full face mask with air breathing apparatus and requires additional training of personnel (23).

The above factors can be overcome if users

Table I - Examples of peptide drugs and manufacturing methods

Peptide	Length	Quantities	Status	Synthetic strategy / Coupling method
Eptifibatide	7	>200 kg	commercial	solution
Leuprolide	9	25-50 kgs	commercial	solution / solid
Pramlintide	37	>10 kgs	III	solution / solid
Exendine	39	NA	III	solid
Zinconotide	25	1-5 kgs	III	solid
Fuzeon (T-20)	36	up to 4 MT	approved	solid/fragment condensation HBTU/HOBt or / Cl-HOBt
Autosiban	8	NA	approved	solution DCC/HOBt
Theratope	43	NA	III	solution/block coupling DCC/HOBt
Thymalfasin	28	NA	III/approval	solid phase
Desmopressin	9	NA	approved	solution DCC / HOBt

comply to transportation and storage regulations and increase their alertness to safety precautions, installation of special protective equipment, as well as working in close contact with the supplier of raw materials and learning from his experience. However, this may affect the manufacturing schedule and production price.

c. Yield of Coupling

As it is obvious, adopting highly effective coupling reagents will result in avoiding double coupling, deletion and modified peptides, will cut the solvent and protected amino acid use, favour the purification step and therefore, decrease production cost.

Today, majority of peptide drugs up to 13-15 amino acids are synthesized using solution approach, Boc chemistry and conventional DCC/HOBt method. Recently we observe a shift of industry to solid phase Fmoc chemistry, increasing in use of modern coupling techniques (HBTU/TBTU and HCTU/TCTU) together with fragment condensation approach of the 12-15 amino acid sequences (1,3,24-26).

CONCLUSIONS

The formation of the amide bond together with chirality of the molecules plays a key role in the preparation of a broad range of organic compounds. It begins with the small molecules through the peptides to fully synthetic proteins. Today, the synthesis of peptide or protein based pharmaceutical drug requires up to 100 steps, the name of the game is "production cost" and the modern synthetic technologies play a central role in this battle. A thorough study of the mechanism in each method involving basic producer for logistic and technical support is a necessary term for the optimization of the coupling step of a new peptide drug and further up-scaling towards bulk manufacturing. Today, Cl-HOBt as an additive and HCTU/TCTU are excellent alternatives to the most classic methods for the large scale manufacturing of peptide and proteins.

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Peptide Synthesis

Coupling Reagents and Additives

In addition to standard coupling reagents such as DCC **36650**, EDAC.HCl **39391**, CDI **21860** and additives, Fluka offers a broad range of phosphonium, uronium and thiouronium based coupling reagents for peptide synthesis. They have many advantages over the standard reagents (e.g. carbodiimides plus an additive, reagents for preparing mixed anhydrides, etc.):

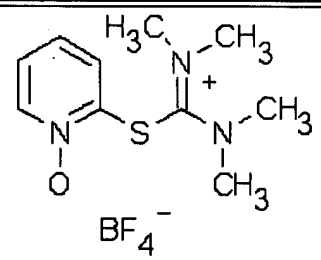
- simple reaction conditions
- very short reaction times
- high yields of product
- very low racemization
- high yield with N-methyl amino acids

BOP **12802** is a widely used reagent for peptide synthesis [1]. Analogues of the BOP reagent containing a uronium group such as HBTU **12804** [2], TBTU **12806** [3], HATU **11373** [4] HBPyU **12809** [5] and HBPipU **12792** have been introduced. PyBroP **18565** [6] is suitable for the coupling of α,α -dialkyl amino acids. The pyrrolidine analogues of phosphonium and uronium reagents [N,N ,N',N-bis(tetramethylene) derivatives] are superior as no carcinogenic by-products are produced. TDBTU **37345** [3], TPTU **37347** [3], HPPyU **37346** [7] and TOTU **02580** [8] are recommended for fragment condensation and other critical cases leading to minimal racemization.

BroP **18570** [9], PyCloP **26564**[10], PyCIU **23955** [10], PipCIU **23953** and Chloro-N, N, N',N'-bis (tetramethylene)formamidinium tetrafluoroborate **23957** [10] are extremely reactive and suitable for coupling N-methyl amino acids. 2-Chloro-1,3-dimethylimidazolidinium hexafluorophosphate **24375** [11] is used for the coupling of FMOC-amino acids to Wang resin (benzyl alcohol resin). 2-Chloro-1,3-dimethylimidazolidinium tetrafluoroborate **24377** [12] is a reagent for the esterification of C-terminal amino acids to Wang resin without racemization and for coupling α,α -dimethyl amino acids.

In addition to our really comprehensive portfolio of uronium- and phosphonium-type coupling reagents, Fluka now likes to introduce a **NEW** thiouronium coupling reagent for efficient peptide coupling and amidation. [13,14]
S-(1-Oxido-2-pyridyl)-N,N,N',N'-tetramethylthiuronium tetrafluoroborate (**TOTT**) is ideally suited for rapid and high-yielding preparation of primary amides when reacted with carboxylic acids and ammonium chloride in the presence of diisopropylethylamine.

Table 1. Thiouronium Peptide Coupling Reagent, TOTT

Product No.	Product Name	Structure
94623	S-(1-Oxido-2-pyridyl)-N,N,N',N'-tetramethylthiuronium tetrafluoroborate (**TOTT**) purum >95%(NMR)	

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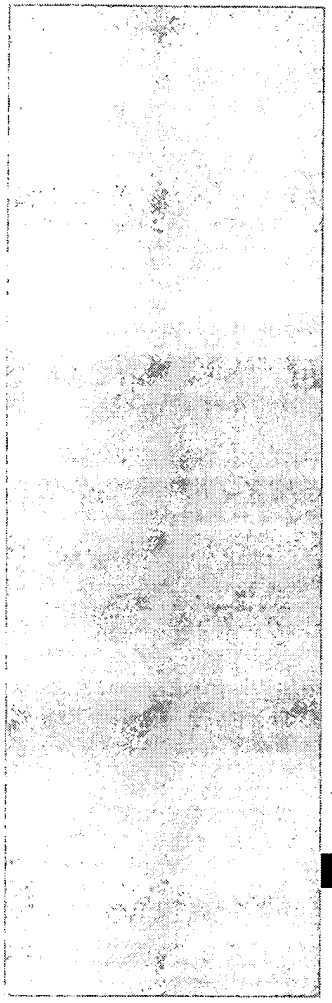

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